Docket No. 01416/OP551-PC-US

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

Koichi ISHIGURO, et al : EXAMINER: S. Turner

SERIAL NO. 09/142,613 :

FILED: APRIL 19, 1999 : GROUP ART UNIT: 1647

#26
M.G.J
12/3/02

FOR: ANTI-PHOSPHORYLATED TAU PROTEIN ANTIBODIES AND METHODS FOR DETECTING ALZHEIMER'S DISEASE WITH THE USE OF THE SAME

DECLARATION UNDER 37 C.F.R. 1.132

HONORABLE COMMISSIONER OF PATENTS & TRADEMARKS
WASHINGTON, D.C. 20231

SIR:

I, Koichi Ishiguro who deposes and states that:

1. I am a graduate of the University of Tokyo, Japan, where I majored in biochemistry, and where I received my doctorate with a specialization in Biochemistry.

Considered
01-29-03

2. I have been employed by Mitsubishi Kagaku Institute of Life Sciences, Machida-shi, Tokyo, Japan for 13 years as a researcher in the field of biochemistry.

3. I am one of inventors of the above-identified application.

4. I have read and understood the Office Action dated May 9, 2001 and the documents cited therein.

5. The following experiments were conducted by me or under my direct supervision and control.

We describe the difference of two antibodies (anti-PS199 and AT8) as below.

1. Summary

(1) Epitopes are completely different. Anti-PS199 binds to phosphorylated serine at position 199. It binds to phosphorylated tau peptide (PS199), paired helical filament (PHF) tau-protein, brain extract of the patients suffered Alzheimer's disease (AD) and that of rat. AT8 does not bind to phosphorylated tau peptide (PS199). It binds to PHF-tau protein only.

(2) The results of cerebrospinal fluid (CSF) analysis (with use of enzyme immunoassay, EIA) are completely different. The assay was done by the same procedure with the same materials (without the probing antibodies AT8 and anti-PS199) and the same samples (CSF).

PS199 assay can distinguish AD patients from control (CTL) (AD>CTL), on the other hand, AT8 assay can not.

2. Introduction

The examiner rejected the claims of our submitted US patent proposal on diagnostic method of Alzheimer's disease by measuring phosphorylated tau in cerebrospinal fluid (CSF) under 35 U.S.C. 102(b). Among the basis for the rejection, the examiner pointed out the reference of Vandermeeren et al., J. of Neurochemistry 61: 1828-34 (1993) [1], that teaches detection of tau proteins in CSF with a sensitive sandwich EIA. The sandwich assay utilizes monoclonal antibody AT8 which was formerly believed to recognize abnormally phosphorylated serines 199-202 in tau, but later found to recognize tau doubly phosphorylated at Ser202 and Thr205 [2]. Independently, my colleagues and I determined phosphorylation sites on PHF-tau [3] and prepared several antibodies against phosphorylation sites including anti-PS199 that recognizes tau phosphorylated at Ser199 [4]. We developed sandwich EIA with anti-PS199.

Here, we confirm the difference between anti-PS199 and AT8 in antigen specificity, and indicate more usefulness of anti-PS199 than AT8 for detection of phospho-tau in CSF.

3. Methods

(1) Dot blot analysis of the antibodies against phosphorylation sites on tau.

Each chemically synthesized peptide containing amino acid sequence found in tau protein was spotted on Immobilon-P membrane (Millipore). The peptide spots were reacted with anti-PS199 or AT8. The reaction was detected using alkaline phosphatase conjugated anti-IgG antibody with NBT and BCIP as chromogens. As positive controls containing phosphorylated tau, we also spotted PHF (20.9 fmol tau), rat neonatal brain extract (460 fmol tau) and SDS-insoluble fraction from AD brain extract containing PHF. Based on the report

that AT8 recognizes double phosphorylation sites at Ser202 and Thr205, we also compared immunoreactivities of anti-PS202 and anti-PT205 with that of AT8. The amino acid sequence of each peptide is indicated in the patent application document and our publication [4].

(2) Enzyme immunoassay, EIA

AT8 and anti-PS199 were biotinylated with sulfo-NHS-LC biotin under the instruction (Pierce). Using a plate coated with anti-human tau monoclonal antibody, HT7, we measured phosphorylated tau levels in CSF with PHF as a standard. Reacted biotinylated antibody was detected by peroxidase-conjugated streptoavidin and 3,3',5,5'-tetramethyl benzidine (TMB) as a chromogen.

4. Results

(1) Dot blot analysis

Anti-PS199, anti-PS202 and anti-PT205 reacted with its antigen phosphopeptides, respectively, but not with a peptide K2 which contains non-phosphorylated Ser199, Ser202 and Thr205. Anti-PS202 also reacted with PS199,202, another peptide phosphorylated at Ser202 as well as at Ser199. The result indicates that each antibody described above is specific to each phosphorylation site. On the other hand, AT8, a monoclonal antibody to PHF, could not recognize these phosphopeptides and non-phosphopeptides indicated in Fig. 1A. AT8 did not also bind to ten-fold amount of these peptides. The comparison clearly indicates that AT8 is different from anti-PS199, anti-PS202 and anti-PT205.

(2) EIA measurement of phospho-tau levels

Calibration curves of phospho-tau (PHF) levels were obtained by EIA. Detection limits were set up at the mean + 3 SD of OD at the zero standard. In the case of AT8, the OD of phospho-tau level in CSF was under the detection limit, as mentioned by Vandermeeren et al. [1]. On the other hand, anti-PS199 can detect phospho-tau in CSF, showing the superiority to AT8.

5. Discussion

Antigen specificity of AT8 is different from that of anti-PS199 prepared by us. AT8 was reported to recognize double phosphorylation sites at Ser202 and Thr205 [2]. Although the double phosphopeptide was not available for us, we confirmed that AT8 is different from anti-PS202 and anti-PT205, each of which binds to the corresponding single phosphopeptide. These phosphorylation sites were determined by us [3], independently of Vandermeeren et al.

AT8 is well-known and commercially available monoclonal antibody for detecting

PHF-tau. But AT8 could not detect phospho-tau in CSF, as reported by Vandermeeren et al. [1] and confirmed by us in this report. It is because tau in CSF is not phosphorylated at AT8 site, or because the AT8 region is not present or available for immunoreactivity with AT8.

We can measure phospho-tau level in CSF by the EIA with anti-PS199 and show that the levels increase in Alzheimer's disease. Our priority is found here. We developed the detection system independently of Vandermeeren et al. Their paper only teaches CSF-tau measurement for AD diagnosis, but their detection of phospho-tau in CSF was unsuccessful. They also reported that AT8 recognized phosphorylated serines 199-202, but could not pointed out which serine was good for the diagnosis. We detected phospho-serine 199 in AD CSF, and found that PS199 is a better marker for AD diagnosis [5] than CSF-tau reported by Vandermeeren et al. [1], indicating that our EIA is more progressive and useful than theirs.

6. References

[1] Vandermeeren, M., Mercken, M., Vanmechelen, E., Six, J., Van de Voorde, A., Martin, J.-J. and Cras, P. Detection of τ proteins in normal and Alzheimer's disease cerebrospinal fluid with a sensitive sandwich enzyme-liked immunosorbent assay. J. Neurochem. 61, 1828-1834 (1993).

[2] Goedert, M., Jakes, R. and Vanmechelen, E. Monoclonal antibody AT8 recognises tau protein phosphorylated at both serine 202 and threonine 205. Neurosci. Lett. 189, 167-169 (1995).

[3] Ishiguro, K., Omori, A., Takamatsu, M., Sato, K., Arioka, M., Uchida, T. and Imahori, K. Phosphorylation sites on tau by tau protein kinase I, a bovine derived kinase generating an epitope of paired helical filaments. Neurosci. Lett. 148, 202-206 (1992).

[4] Ishiguro, K., Sato, K., Takamatsu, M., Park, J.-M., Uchida, T. and Imahori, K. Analysis of phosphorylation of tau with antibodies specific for phosphorylation sites. Neurosci. Lett. 202, 81-84 (1995).

[5] Ishiguro, K., Ohno, H., Arai, H., Yamaguchi, H., Urakami, K., Park, J.-M., Sato, K., Kohno, H. and Imahori, K. Phosphorylated tau in human cerebrospinal fluid is a diagnostic marker for Alzheimer's disease. Neurosci. Lett. 270, 91-94 (1999).

FIGURE LEGENDS

Fig. 1 Dot blot

Each peptide was dotted on the position indicated in panel A. The dotted amount was 10 pmol except that in panel C where 100 pmol was used. The membrane was blotted by

AT8 (800 ng/ml, B and C), anti-PS199 (140 ng/ml, D), anti-PS202 (800 ng/ml, E) or anti-PT205 (44 ng/ml).

Fig. 2 EIA measurement of phospho-tau levels

Calibrations of PHF-tau in sandwich EIA with anti-PS199 (a) and AT8 (b). All dilutions were tested in triplicate. Detection limits were set up at the mean + 3 SD of OD at the zero standard. Phospho-tau levels in CSF were measured by the sandwich EIA with anti-PS199 (c) and AT8 (d). Each dashed line indicates its detection limit. Bars show mean values.

6. I hereby declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of application or any patent issuing thereon.

Date: March 4 , 2002

Koichi Ishiguro

Koichi Ishiguro, Ph.D.